PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Sojiro SHIOKAWA, *et al.*

Docket No: Q64460

Appln. No.: 09/856,372

Group Art Unit: 1624

Confirmation No.: 8305

Examiner: Brenda Libby COLEMAN

Filed: November 2, 2001

For: BENZOXAZOLE DERIVATIVES AND MEDICAMENTS COMPRISING SAID DERIVATIVES AS ACTIVE INGREDIENT

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Yasuo SATO, hereby declare and state:

THAT I am a citizen of Japan;

THAT I graduated from The University of Tokyo, Faculty of Pharmaceutical Science, in March 1985, received a Master's degree from The University of Tokyo in March 1987, and received the degree of Doctor of Philosophy (pharmaceutical science, especially organic and medicinal chemistry) from The University of Tokyo in May 1995.

THAT I have been employed by Meiji Seika Kaisha, Ltd. since April 1987, where I have been engaged in the research and development of new drugs;

THAT I am a co-author of each of the publications listed in the Appendix attached hereto; and

THAT I am familiar with the prosecution of the above-identified U.S. patent application, including the Office Action mailed September 13, 2004, and the Interview Summary, Form PTOL-413, provided by the Examiner to Counsel after the Examiner Interview conducted November 16, 2004.

RULE 132 DECLARATION
U.S. Appln. No. 09/856,372

I provide the following information so that the Examiner may place the results from Test Examples 2 and 4 and the tables at pages 38 and 40 of the specification in a context that will allow her to appreciate the significance of the values reported in the tables.

First, with regard to Test Example 2, the experimental results shown in the table at page 38 of the specification were obtained by experimental results below.

The following table indicates the results of inhibitory action on rat diarrhea under restriction stress. In the table, each result is indicated as numerator/denominator, wherein the numerator indicates the number of rats with diarrhea and the denominator represents the number of rats applied with restriction stress. When rats were administered solely with vehicle (a solvent) under the restriction stress, five rats among eight tested rats suffered from diarrhea. When rats were administered solely with vehicle without the restriction stress, no diarrhea was observed among eight tested rats.

Test Substance	Dose (mg/kg)						
	0.0003	0.003	0.03	0.1	0.3	1	3
Example 1(b)	4/8	1/8	1/8		0/8		0/8
Hydrochloride of C			3/8	4/8	2/8	2/8	1/8
Hydrochloride of E		4/8	2/8		0/8		1/8
G (Granisetron)			4/8	3/8	0/8	1/8	

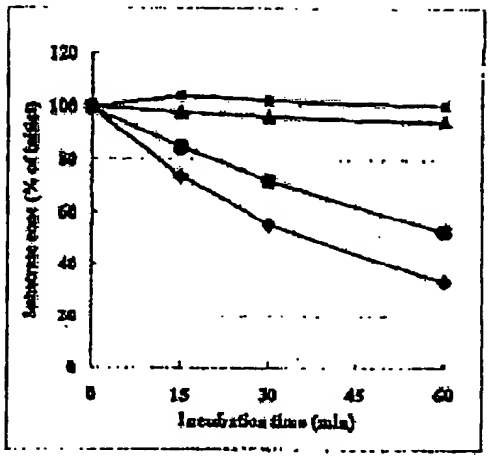
The compound of Example 1(b) gave only one rat diarrhea (1/8) at the dose of 0.003 mg/kg, while the comparative substance (hydrochloride of E) gave four rats diarrhea (4/8) at the dose of 0.003 mg/kg. Furthermore, the comparative substance (hydrochloride of E) gave two rats diarrhea (2/8) even at 0.03 mg/kg, which is a tenfold higher dose than the 0.003 mg/kg dose of the compound of Example 1(b), which showed almost complete (1/8) suppressing effect on the diarrhea caused by the restriction stress. Accordingly, these results demonstrate an unexpectedly higher suppressing effect of the compound of the present invention as compared to the prior disclosed compounds.

In addition, I have found a publication, "The Journal of Pharmacology and Experimental Therapeutics," 261, pp. 297-303, 1992, which discloses, in Fig. 3 on page 299, the results of suppressing effect on diarrhea of granisetron and ondansetron obtained by similar experiments to those applied in Test Example 2 of the present application. A copy of the publication is being submitted herewith.

In the publication, ED_{50} values are 354 μg/kg for ondansetron and 142 μg/kg for granisetron, which are around 50% in the longitudinal axis, and this 2.5-fold lower ED_{50} of granisetron than ondansetron is considered by the authors as evidence of higher effectiveness of granisetron when inclinations of the plots are taken into consideration. Accordingly, the 16-fold lower ED_{50} value of the compound of Example 1(b) of the present invention as compared to that of the comparative substance can be understood by one of ordinary skill in the art as indicating that the compound of the present invention has significantly higher suppressing effect on diarrhea than the comparative compound.

With respect to Test Example 4, the results obtained by the experiment are shown in the following table and figure. The metabolic elimination of test compounds were evaluated after incubation with human liver S) fraction. In the experiment, incubation was carried out in the presence of NADPH-generating system, and the initial substrate concentration was 50 μ mol/L. In the table below, metabolic activity is shown as nmol/min/mg protein, and the symbol "N.D." is an abbreviation of "not detected."

Incubation time	Substrate concentration (% of initial)			
(min)	Compound of Example 1(b)	Hydrochloride of C	Hydrochloride of D	Hydrochloride of E
0	100.0	100.0	100.0	100.0
15	104.2	98.3	74.0	85.0
30	102.6	96.6	55.6	72.2
60	100.7	94.6	33.5	52.9
Metabolic activity	N.D.	0.08	0.87	0.60



Square: Compound of Example 1(b)
Triangle: Hydrochloride of C
Diamond: Hydrochloride of D
Circle: Hydrochloride of E

The above results clearly demonstrate that the hydrochloride of E and the hydrochloride of D rapidly disappear metabolically, while the compound of the present invention (compound of Example 1(b)) and the hydrochloride of C are metabolically very stable which gave very slow degradation by metabolism. This experiment was carried out by using a pooled human liver S9 fraction and thus the experiment was conducted under conditions of n=1. However, I conducted several separate repetitions of the same experiments according to the method of Test Example 4 and found that the experimental results obtained were highly reproducible. Accordingly, I concluded that the compound of the present invention has higher metabolic stability than the hydrochloride of E.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 01, 18, 2005

Dr. Yasuo SATO

APPENDIX

Endo, Y.; Sato, Y.; and Shudo, K. "Synthesis of 7-Substituted Indolactam-V. An Introduction of Hydrophobic Moieties on the Indole Ring," *Tetrahedron*, 43, pp. 2241-2247, 1987;

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Role of the Serotonin$_3$ Receptor in Stress-Induced Defecation

KEIJI MIYATA, TAKESHI KAMATO, AKITO NISHIDA, HIROYUKI ITO, HIDENOBU YUKI, MAYUMI YAMANO, RIE TSUTSUMI, YOSHINORI KATSUYAMA and KAZUO HONDA

Medicinal Research Laboratories I, Central Research Laboratories, Yamanouchi Pharmaceutical Co. Ltd., 21 Miyukigaoka, Tsukuba City, Ibaraki 305, Japan

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ABSTRACT

The possibility that 5-hydroxytryptamine (serotonin; 5-HT) mediates bowel dysfunction caused by stress was evaluated in rats and mice treated with 5-HT or thyrotropin-releasing hormone (TRH) injection and in rats subjected to stress. Restraint stress at room temperature (23°C) significantly increased fecal pellet output without the formation of gastrointestinal mucosal lesions in free-feeding rats, and caused diarrhea in 90 to 100% of animals within 3 hr in food-deprived rats. Oral YM060, ondansetron, granisetron, atropine and diazepam and s.c. tetrodotoxin inhibited these stress-induced changes in bowel function in fed and fasted rats. ED_{50} values were 1.1 (0.2–6.6) and 2.5 (1.1–5.7) μg/kg for YM060, 483 (338–691) and 354 (262–477) μg/kg for ondansetron, 208 (111–393) and 142 (48.9–414) μg/kg for granisetron, 811 (639–1,030) and 847 (641–1,118) μg/kg for atropine, 3,099 (1,499–6,405) and 5,396 (4,768–6,106) μg/kg for diazepam and 1.9 (1.7–2.1) and 3.3 (1.6–6.5) μg/kg for tetrodotoxin, respectively. Methysergide inhibited stress-induced diarrhea with an ED_{50} value of 724 (384–1,366) μg/kg s.c., whereas it had partial effect on stress-induced increases in fecal pellet output. Exogenous 5-HT increased fecal pellet output in rats and caused diarrhea in mice. YM060, granisetron, atropine and tetrodotoxin but not methysergide dose-dependently inhibited both 5-HT, 10 mg/kg s.c.-induced increases in fecal pellet output and 5-HT, 3 mg/kg i.p.-induced diarrhea, with ED_{50} values of 4.4 (3.8–5.1) and 10.6 (6.7–16.8) μg/kg p.o. for YM060, 309 (171–561) and 387 (163–918) μg/kg p.o. for granisetron, 1,108 (670–1,833) and 3,771 (2,368–6,004) μg/kg p.o. for atropine and 2.8 (1.6–4.2) and 5.0 (3.0–8.3) μg/kg s.c. for tetrodotoxin, respectively. Subcutaneous TRH, an endogenous candidate in centrally mediated stress-induced bowel function responses, increased fecal pellet output at doses of 3 to 100 mg/kg in rats. The change in bowel function induced by TRH (10 mg/kg s.c.) was also reduced by p.o. YM060, granisetron and atropine and s.c. tetrodotoxin with ED_{50} values of 11.6 (4.4–31.1), 131 (52.3–326), 1,606 (995–2,594) and 3.1 (2.7–3.6) μg/kg, respectively. In contrast, methysergide (300–3,000 μg/kg s.c.) did not affect TRH-induced defecation. These data demonstrate that exogenous and endogenous 5-HT, whose release may be induced by TRH, appear to cause an increase in the number of stools excreted or diarrhea in rats or mice *via* the 5-HT$_3$ receptor. Moreover, these data also suggest that endogenous 5-HT may be one of the substances that mediate stress-induced responses of gastrointestinal function.

Endogenous 5-HT is found in the blood platelets, the nervous system and the gut, and approximately 90% is estimated to be within the gastrointestinal tract. Exogenous 5-HT is known to evoke various gastrointestinal disorders such as emesis, diarrhea, visceral pain and motility disorders (King and Sanger, 1989; Kilpatrick *et al.*, 1990). It is therefore suggested that 5-HT has a relationship to gut function. Some studies have revealed an association between the occurrence of stressful experiences and the appearance of disturbances in bowel function (Yano *et al.*, 1978; Gue *et al.*, 1987; Bueno and Gue, 1988; Williams *et al.*, 1988), indicating that gut function is affected by various stresses.

Recently, several selective 5-HT$_3$ receptor antagonists including ICS205–930, ondansetron (GR38032F), granisetron (BRL43694) and zacopride have been developed (Richardson *et al.*, 1985; Butler *et al.*, 1988; Smith *et al.*, 1988; Sanger and Nelson, 1989). Many actions attributable to the 5-HT$_3$ receptor have been described in both the peripheral and central nervous systems, and clinical trials are already showing the potential use of these 5-HT$_3$ receptor antagonists in a number of disorders of the gastrointestinal tract and central nervous system (King and Sanger, 1989; Kilpatrick *et al.*, 1990).

YM060, a tetrahydrobenzimidazole derivative, has been shown by *in vitro* blockade of 5-HT- or 2-methyl-5-HT-induced contractions in the guinea pig colon (Miyata *et al.*, 1991a) and *in vivo* blockade of 5-HT-induced transient bradycardia in rats (Miyata *et al.*, 1991b) to be a potent and selective 5-HT$_3$ receptor antagonist. The role of 5-HT$_3$ receptors in controlling gastrointestinal contractility *in vitro* is well established. Administration of 5-HT or the selective 5-HT$_3$ receptor agonist

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); TRH, thyrotropin-releasing hormone; PG, prostaglandin; IBS, irritable bowel syndrome; CRF, corticotropin-releasing factor; EC:, enterochromaffin.

2-methyl-5-HT to guinea pig ileum (Buchheit *et al.*, 1985; Butler *et al.*, 1988) or colon (Grossman *et al.*, 1989; Miyata *et al.*, 1991a) stimulates contractile responses which are sensitive to 5-HT_3 receptor antagonists.

In the present study we examined the effects of 5-HT and TRH on colonic function *in vivo* to investigate the possibility that 5-HT acts as a mediator of restraint-stress induced gut dysfunction. We also determined the 5-HT receptor subtype associated with responses to 5-HT.

Methods

Animals. Male Wistar rats weighing 180 to 320 g and male ICR mice weighing 27 to 38 g were used. The animals were maintained on ordinary laboratory chow and tap water *ad libitum* under a constant 12-hr light-dark cycle. In the fasted condition, they were deprived of food overnight before the experiments but allowed free access to water.

Restraint stress on defecation in rats. The stress model used in all experiments was restraint stress. Animals were stressed by placing them in individual compartments of special stress cages (Natsume Seisakusho Co. Ltd., Tokyo, Japan; KN-468, W265 × L95 × H200 mm) at room temperature (23°C).

In the first series of experiments, fecal pellet output induced by restraint stress was observed using animals that were not deprived of food before testing. The significant increase in fecal pellet output lasted for 6 hr during stressing (see "Results"). Because the changes in fecal pellet output during the 1st hr after restraint were marked, the effects of the test drugs on stress-induced increases in pellet output were determined at this time point.

In the second series, restraint stress-induced diarrhea was observed in overnight fasted animals. Ninety to 100% of rats subjected to this stress developed diarrhea within 3 hr. The effects of the test drugs were therefore determined on diarrhea induced by restraint for 3 hr. Diarrhea was defined as wet, unformed stools and scored as present or absent for each animal. From these data, the incidence of diarrhea (number of rats with diarrhea/total number of rats tested) was calculated.

In each experiment, the test drugs were given p.o. 1 hr or s.c. 30 min before stress.

Effect of 5-HT on defecation in rats. Initial experiments were conducted in the fed rat to determine the effects of 5-HT administration on defecation. Preliminary experiments showed 5-HT to increase fecal pellet output. To determine doses for the following studies, the dose-response curve for 5-HT or 2-methyl-5-HT-induced fecal pellet output was therefore determined using approximately 3-fold increases in dose of each agonist. Because the effects of 5-HT and 2-methyl-5-HT on defecation lasted for approximately 1 hr, the number and weight of fecal pellets expelled by each animal was measured 1 hr after 5-HT or 2-methyl-5-HT injection. Inasmuch as the effect of 5-HT on fecal pellet output was approximately 3 times greater than that of 2-methyl-5-HT (see "Results"), we tested the ability of several drugs from different chemical classes to block the response to a 10-mg/kg dose of 5-HT. Test drugs were administered p.o. or s.c. 1 hr or 30 min before 5-HT administration.

Effect of TRH on defecation in rats. The effect of TRH on defecation was also evaluated using fed rats. Subcutaneous administration of TRH, like 5-HT, resulted in increased fecal pellet output. Dose-response curves for the TRH-induced increase in stools was therefore conducted by approximately 3-fold increases in TRH dose (1–100 mg/kg). Because the effect of TRH on defecation lasted for 3 to 4 hr, the number of fecal pellets expelled by TRH was measured 4 hr after TRH injection. Inhibitory activity of the test drugs was evaluated in TRH (10 mg/kg s.c.)-induced fecal pellet output. Test drugs were given p.o. 1 hr or s.c. 30 min before TRH administration.

5-HT-, PGE_2- or castor oil-induced diarrhea in mice. Food and water were provided *ad libitum* to mice before the experiments. Diarrhea was induced by administration of 5-HT (3 mg/kg i.p.), PGE_2 (300 μg/kg i.p.) or castor oil (0.3 ml/mice p.o.). After injection, mice were placed into individual observation cages lined with absorbent paper. Animals were observed for 3 hr for the occurrence of diarrhea. Diarrhea was defined as wet, unformed stools and scored as present or absent for each animal. From these data, the incidence of diarrhea (number of mice with diarrhea/total number of mice tested) was calculated as a percentage. Test drugs were given p.o. 1 hr or s.c. 30 min before administration of 5-HT, PGE_2 or castor oil.

Statistical evaluation. Because there was no significant difference between mean of the weight per fecal pellet excreted by stress or 5-HT and that excreted in control rats, the number of pellets was counted in the present study. Values for fecal pellet output represent mean of the number of pellets ± S.E.M., whereas those for diarrhea represent the percentage of incidence. Statistical significance of values for fecal pellet output was determined by analysis of variance (Kruskal-Wallis H test) and differences between treatment groups were compared by the Wilcoxon-multiple-comparison test or the Mann-Whitney U test. Statistical significance of values for diarrheal incidence was determined by the Fisher exact probability test. Probabilities of $< 5\%$ ($P < .05$) were considered significant. ED_{50} values with 95% CL were calculated as the dose causing 50% inhibition of the increase in stools excreted or the occurence of diarrhea (control) by log-probit analysis from data obtained for three to four doses of each compound. All calculations were determined with reference to concomitantly tested control animals.

Drugs. YM060, [(*R*)-5-[(1-methyl-3-indolyl)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride], ondansetron [GR38032F; (±)-1,2,3,9-tetrahydro-3-[(methylimidazol-1-yl)methyl]-9-methyl-4H-carbazol-4-one hydrochloride], granisetron [BRL43694; N-endo-9-methyl-9-azabicyclo [3.3.1]non-3-yl)-1-methyl-indazole-3-carboxamide hydrochloride], 2-methyl-5-HT and diazepam were prepared by Yamanouchi Pharmaceutical Co. Ltd, Tsukuba City, Ibaraki, Japan. Methysergide hydrogen maleate was kindly donated by Sandoz Ltd (Basle, Switzerland). 5-HT creatinine sulfate was obtained from E. Merck (Darmasadt, Germany). Tetrodotoxin, loperamide hydrochloride and PGE_2 were from Sigma Chemical Co. (St. Louis, Mo). Atropine sulfate, TRH and castor oil were purchased from Wako Pure Chemical Industries (Osaka, Japan), Peptide Institute Inc. (Osaka, Japan) and Nakalai Tesque Inc. (Kyoto, Japan), respectively. All drug doses are given as the free base. YM060, ondansetron, granisetron, atropine, diazepam and loperamide were suspended with 0.5% methylcellulose solution. Methysergide was dissolved in 0.1 N HCl solution, and the pH of the solution was adjusted to 6 with solid $NaHCO_3$. 5-HT, 2-methyl-5-HT, TRH, tetrodotoxin and PGE_2 were dissolved in physiological saline. All drugs were administered to rats or mice at a volume of 5 or 10 ml/kg, respectively.

Results

Blockade of stress-induced increase in number of stools excreted in fed rats. Restraint stress significantly increased fecal pellet output without the formation of gastrointestinal mucosal lesions in rats allowed to eat and drink *ad libitum*. Figure 1 shows the time course of changes in fecal pellet output produced during restraint stress. Stool excretion did not increase by stress in hr 2. The reason for lack of response is unknown, but may be that the changes in fecal pellet output during the 1st hr after restraint were marked. After p.o. or s.c. administration (1 hr or 30 min pretreatment), all drugs used in the present study dose-dependently inhibited restraint stress-induced increases in stools excreted in fed rats (fig. 2). ED_{50} values for YM060, ondansetron, granisetron, atropine, tetrodotoxin and diazepam were 1.1 (0.2–6.6), 483 (338–691), 208 (111–393) and 811 (639–1,030) μg/kg p.o., 1.9 (1.7–2.1) μg/kg s.c. and 3,099 (1,499–6,405) μg/kg p.o., respectively. Methysergide also significantly inhibited the increases in fecal pellet output in doses up to 1,000 μg/kg s.c., and the degrees of inhibition were 51.0 ± 8.4 and 52.9 ± 6.7% by 1,000

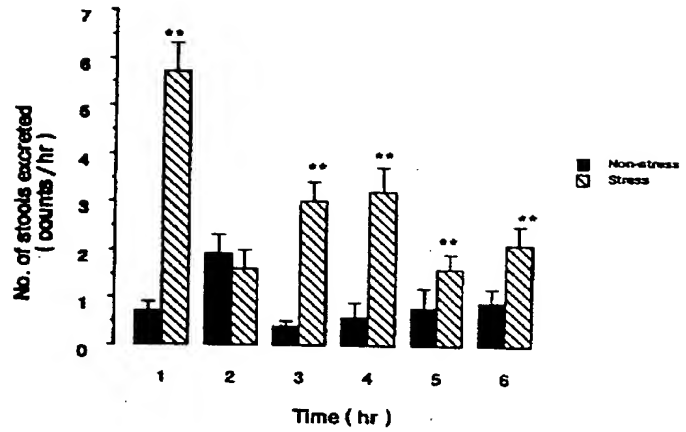

Fig. 1. Effect of restraint stress on stool excretion in fed rats. Each bar represents mean ± S.E.M. for 20 rats. **P < .01 compared with the nonstress control group (Wilcoxon-multiple-comparison test).

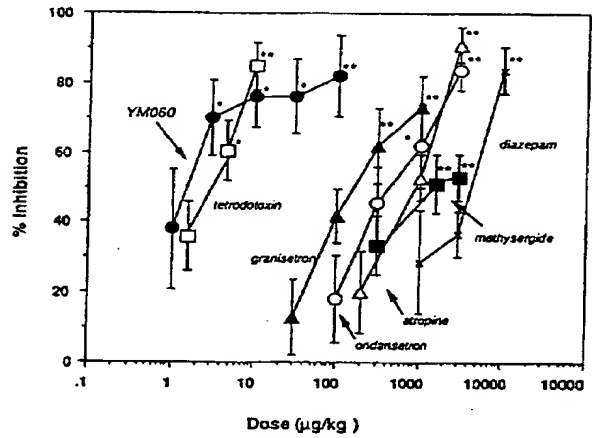

Fig. 2. Effects of YM060 (●), ondansetron (○), granisetron (▲), atropine (△), tetrodotoxin (□), methysergide (■) and diazepam (×) on restraint stress-induced increases in the number of stools excreted in fed rats. Each point represents mean ± S.E.M. for 10 rats. Tetrodotoxin and methysergide were given s.c. 30 min before restraint stress and other drugs were given p.o. 1 hr before restraint. Stool excretion was observed for the first hour of restraint. *P < .05; **P < .01 compared with the control group (Wilcoxon-multiple-comparison test).

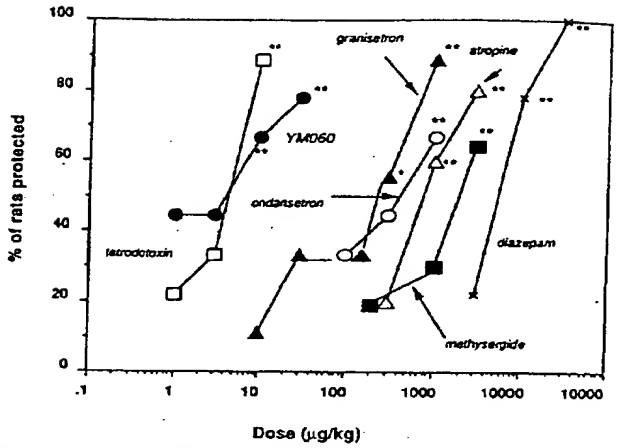

Fig. 3. Effects of YM060 (●), ondansetron (○), granisetron (▲), atropine (△), tetrodotoxin (□), methysergide (■) and diazepam (×) on restraint stress-induced diarrhea in fasted rats. Each point represents the percentage of the incidence of diarrhea calculated from 10 rats. Tetrodotoxin and methysergide were given s.c. 30 min before restraint stress and other drugs were given p.o. 1 hr before restraint. Diarrhea was observed for the first 3 hr of restraint. *P < .05; **P < .01 compared with the control group (Fisher exact probability test).

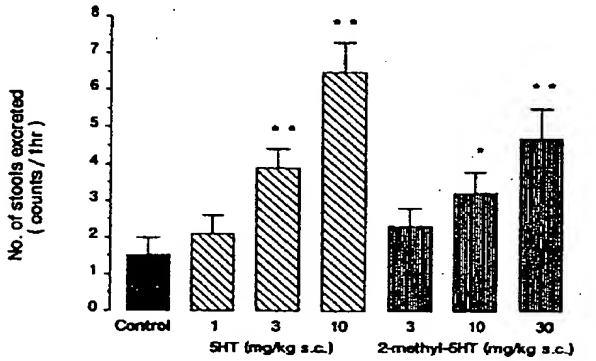

Fig. 4. Effects of 5-HT and 2-methyl-5-HT on stool excretion in fed rats. Each bar represents mean ± S.E.M. for 10 rats. *P < .05; **P < .01 compared with the control group (Wilcoxon-multiple-comparison test).

and 3,000 μg/kg s.c., respectively, indicating that the inhibitory effect of methysergide is not so potent. Tetrodotoxin at doses of 1 and 3 μg/kg s.c. did not affect the condition of rats. But at the highest dose of tetrodotoxin, 10 μg/kg, rats showed depressed condition. So, the inhibitory effect of tetrodotoxin on fecal output may be, in part, due to toxicity.

Blockade of stress-induced diarrhea in fasted rats. In food-deprived rats, restraint stress did not affect fecal pellet output, but caused diarrhea in 90 to 100% of rats within 3 hr. Figure 3 shows that YM060 at p.o. doses of 10 to 30 μg/kg inhibited diarrhea produced by restraint stress in a dose-dependent manner, with an ED_{50} value of 2.5 (1.1–5.7) μg/kg p.o. Ondansetron (1,000 μg/kg p.o.), granisetron (300–1,000 μg/kg p.o.), atropine (1,000–3,000 μg/kg p.o.), tetrodotoxin (10 μg/kg s.c.), methysergide (3,000 μg/kg s.c.) and diazepam (10,000–30,000 μg/kg p.o.) also showed significant preventive effects on stress-induced diarrhea with ED_{50} values of 354 (262–477), 142 (48.9–414), 847 (641–1,118), 3.3 (1.6–6.5), 724 (384–1366) and 5396 (4,768–6,106) μg/kg, respectively.

Effects of 5-HT, 2-methyl-5-HT and TRH on defecation in rats. 5-HT, 2-methyl-5-HT or TRH administered s.c. to the fed rats resulted in a significant change in defecation. An increase in pellet number was induced by 5-HT, 2-methyl-5-HT and TRH in a dose-dependent manner at doses ranging from 3 to 10, 10 to 30 or 3 to 100 mg/kg s.c., respectively (figs. 4 and 5). Neither macroscopic findings of fecal pellets nor the weight of each pellet were affected by these agents (data not shown). Therefore, in the following experiments, only the number of stools excreted by each animal was measured.

Blockade of 5-HT-induced increases in the number of stools excreted in fed rats. As shown in figure 6, YM060 significantly inhibited 5-HT-induced increases in fecal pellet output at an p.o. dose of 10 μg/kg, with an ED_{50} value (95% CL) of 4.4 (3.8–5.1) μg/kg p.o. Granisetron (1,000 μg/kg p.o.), atropine (3,000 μg/kg p.o.) and tetrodotoxin (3–10 μg/kg s.c.) also significantly inhibited 5-HT-induced defecation. The ED_{50} values for granisetron, atropine and tetrodotoxin were 309

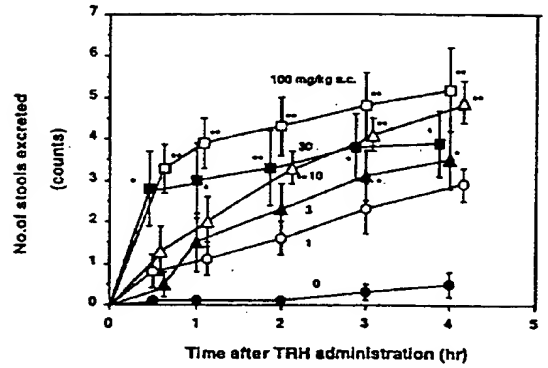

Fig. 5. Effects of varying doses of s.c. administered TRH on stool excretion in fed rats. TRH was administered at time 0. Each point represents mean ± S.E.M. for 10 rats. *P < .05; **P < .01 compared with the control group (Wilcoxon-multiple-comparison test). ●, control; ○, TRH, 1 mg/kg s.c.; ▲, 3 mg/kg s.c.; △, 10 mg/kg s.c.; ■, 30 mg/kg s.c.; □, 100 mg/kg s.c.

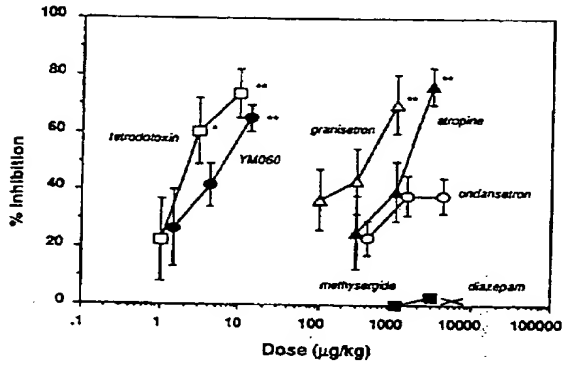

Fig. 6. Effects of YM060 (●), ondansetron (○), granisetron (△) atropine (▲), tetrodotoxin (□), methysergide (■) and diazepam (×) on 5-HT (10 mg/kg s.c.)-induced increases in the number of stools excreted in fed rats. Each point represents mean ± S.E.M. for 10 rats. Tetrodotoxin and methysergide were given s.c. 30 min before 5-HT injection and other drugs were given p.o. 1 hr before 5-HT. Stool excretion was observed for the 1st hr after 5-HT dosing. *P < .05; **P < .01 compared with the control group (Wilcoxon-multiple-comparison test).

(171–561), 1,108 (670–1,833) and 2.8 (1.6–4.2) μg/kg, respectively. Only a small and nonsignificant change was observed with ondansetron (1,000–3,000 μg/kg p.o.). Methysergide (300–3,000 μg/kg s.c.) and diazepam (1,000–10,000 μg/kg p.o.) failed to modify fecal pellet output.

Blockade of TRH-induced increases in the number of stools excreted in fed rats. The effects of drugs on TRH-induced fecal pellet output are shown in figure 7. In control animals, s.c. administration of TRH at 10 mg/kg resulted in an increase in defecation, with a 4-hr pellet output count of 4.9 ± 0.5. YM060 (100 μg/kg p.o.), ondansetron (1,000 μg/kg p.o.), granisetron (300–1,000 μg/kg p.o.), atropine (3,000 μg/kg p.o.) and tetrodotoxin (3–10 μg/kg s.c.) showed significant preventive effects on TRH-induced increases in the number of stools excreted in fed rats, with ED_{50} values of 11.6 (4.4–31.1), 225 (85.5–594), 131 (52.3–326), 1,606 (995–2,594) and 3.1 (2.7–3.6) μg/kg, respectively. On the other hand, methysergide (300–3,000 μg/kg s.c.) and diazepam (1,000–10,000 μg/kg p.o.) did not affect fecal pellet output caused by TRH.

Blockade of 5-HT-, PGE_2- or castor oil-induced diarrhea in mice. Intraperitoneal administration of 5-HT (3 mg/

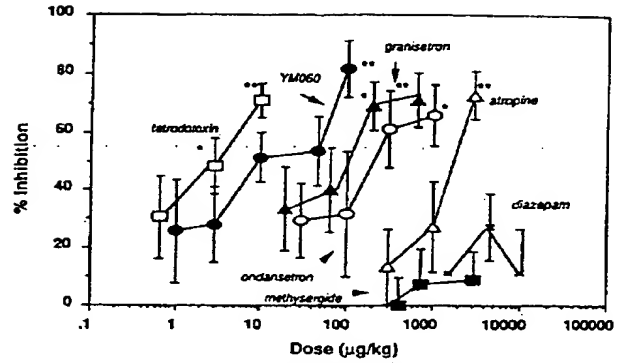

Fig. 7. Effects of YM060 (●), ondansetron (○), granisetron (▲), atropine (△), tetrodotoxin (□), methysergide (■) and diazepam (×) on TRH (10 mg/kg s.c.)-induced increases in the number of stools excreted in fed rats. Each point represents mean ± S.E.M. for 9 to 10 rats. Tetrodotoxin and methysergide were given s.c. 30 min before TRH injection and other drugs were given p.o. 1 hr before TRH. Stool excretion was observed for 4 hr after TRH dosing. *P < .05; **P < .01 compared with the control group (Wilcoxon-multiple-comparison test or Mann-Whitney U test).

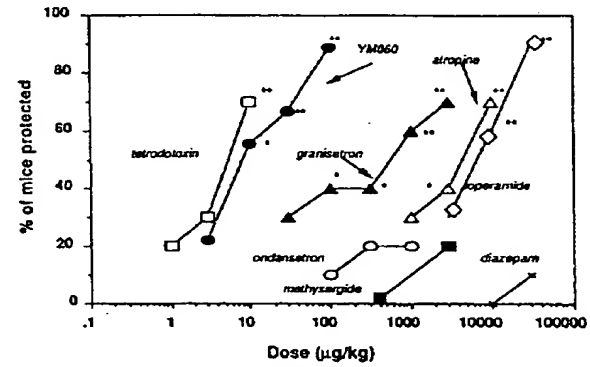

Fig. 8. Effects of YM060 (●), ondansetron (○), granisetron (▲), atropine (△), tetrodotoxin (□), methysergide (■), diazepam (X) and loperamide (△) on 5-HT (3 mg/kg i.p)-induced diarrhea in fed mice. Each point represents the percentage of the incidence of diarrhea calculated from 10 mice. Tetrodotoxin and methysergide were given s.c. 30 min before 5-HT injection and other drugs were given p.o. 1 hr before 5-HT. Diarrhea was observed for the 1st hr after 5-HT dosing. *P < .05; **P < .01 compared with the control group (Fisher exact probability test).

kg) and PGE_2 (300 μg/kg) and p.o. administration of castor oil (0.3 ml/mouse) caused diarrhea in 100% of control (vehicle-treated) mouse. As shown in figure 8, YM060 and granisetron inhibited 5-HT-induced diarrhea with ED_{50} values of 10.6 (6.7–16.8) and 387 (163–918) μg/kg p.o., respectively, but did not affect PGE_2 and castor oil-induced diarrhea at any dose up to 1,000 μg/kg p.o. (data not shown). On the contrary, loperamide, an antidiarrheal agent, produced dose-related decreases in the percentage of mice developing diarrhea induced by 5-HT, PGE_2 and castor oil, with ED_{50} values of 6,339 (5,155–7,795), 3,068 (551–17,085) and 2,699 (2,250–3,238) μg/kg p.o., respectively. Ondansetron showed no significant effects on diarrhea induced by either 5-HT, PGE_2 or castor oil at doses up to 1,000 μg/kg p.o. Atropine and tetrodotoxin inhibited 5-HT-induced diarrhea in mice in doses that decreased 5-HT-induced fecal pellet output in fed rats, with ED_{50} values of 3,771 (2,368–6,004) and 5.0 (3.0–8.3) μg/kg, respectively. Methysergide and diazepam did not affect diarrhea caused by 5-HT at doses up to 3,000 and 30,000 μg/kg, respectively.

Discussion

Stress makes a complex reaction characterized by the activation of both hormonal and neuronal systems. In humans, stress commonly results in gastrointestinal disorders like IBS (Almy, 1973; Thompson, 1984; Narducci *et al.*, 1985), in association with changes in gastrointestinal motility (Yano *et al.*, 1978; Bueno and Gue, 1988), gastric secretion (Kitagawa *et al.*, 1979) and digestive transit (Gue *et al.*, 1987; Williams *et al.*, 1988). In laboratory studies (Wolf, 1981), stress and peptic ulcer formation are well known to be linked. However, the relationship between stress and other bowel function has not been well documented, possibly due to the lack of an appropriate experimental model.

Animal models of bowel dysfunction associated with stress induced by cold, acoustic, ether, cold restraint and wrap restraint have been reported. In general, these stressors are involved in changes in gastric emptying, intestinal transit and/or fecal pellet output. Among these models of stress, cold or cold restraint stress is involved in the genesis of gastric lesions (Brodie, 1962; Kitagawa *et al.*, 1979; Basso *et al.*, 1988), acoustic stress does not affect intestinal transit (Gue *et al.*, 1987), and wrap restraint stress seems to be complicated (Williams *et al.*, 1987, 1988). In the present study, rats that were not deprived of food before testing were subjected to stress by restraint alone at room temperature, resulting in a lasting increase in fecal pellet excretion without the formation of gastrointestinal lesions. Furthermore, in food-deprived rats, restraint stress caused diarrhea without affecting fecal pellet output. In healthy humans, stress increases propulsive motor activity of the colon and causes diarrhea (Almy, 1973; Narducci *et al.*, 1985). In patients with functional disorders of the gut, *e.g.*, IBS, the bowel is hypersensitive to many different types of stimulation and emotional stress often results in abdominal pain and diarrhea (Thompson, 1984). At present, there are few appropriate animal models for experimental diarrhea associated with stress. The restraint-only stress used in the present study is a mild, nonulcerogenic stressor which reproduces the symptoms associated with stress-related bowel dysfunction in humans, suggesting that it may be an appropriate model for the study of the effects of stress on the gastrointestinal tract. Diazepam has been reported to block the increase in colonic motility during exposure to stressful situations in humans (Narducci *et al.*, 1985). A direct central inhibitory effect of diazepam on stress-induced CRF release has been proposed (Ninan *et al.*, 1982). In the present study, diazepam did not affect peripherally administered 5-HT- or TRH-induced bowel dysfunction, but inhibited stress-induced increases in fecal pellet output and diarrhea, confirming that restraint stress-induced changes in bowel function may be evoked centrally.

Several hypotheses may be proposed to explain the effects of various stresses on the gut. They may result from a dominant vagal excitatory activity that occurs after stress exposure. However, the neurohormonal mediators responsible for such activation and the etiology of bowel dysfunction due to stress are not well known. Various stress conditions have been shown to produce the release of endogenous substances such as catecholamines (Axelrod and Reisine, 1984), β-endorphine (Cohen *et al.*, 1981; Williams *et al.*, 1988), CRF (Nakane *et al.*, 1985; Williams *et al.*, 1987; Hashimoto *et al.*, 1988), TRH (Koivusalo and Leppaluoto, 1979; Arancibia *et al.*, 1983; Kubek and Sattin, 1984) and 5-HT (Sharma and Dey, 1981; Horita and Carino, 1982; Ahlman and Dahlstrom, 1983; Horita *et al.*, 1985). The majority of endogenous 5-HT is estimated to be contained within the gastrointestinal tract, particularly within EC cells of the gut mucosa, and 5-HT-containing neurons have been identified in the enteric nervous system of several species (Costa *et al.*, 1982). Exogenous 5-HT is known to evoke various gastrointestinal disorders (King and Sanger, 1989; Kilpatrick *et al.*, 1990) at least in part *via* smooth muscle contraction. Furthermore, the fact that immobilization stress results in an increase in plasma and brain 5-HT concentration in rats (Sharma and Dey, 1981) indicates the possibility that 5-HT acts as a mediator of gut dysfunction caused by stress.

In the present study, both 5-HT and 2-methyl-5-HT, the selective 5-HT$_3$ receptor agonist, caused an increase in the number of fecal pellets excreted in rats. The effect of 5-HT on defecation was approximately 2.5 times as potent as that of 2-methyl-5-HT. This ratio corroborates those obtained with contractile responses in the isolated guinea pig colon (Miyata *et al.*, 1991a) and bradycardic responses in anesthetized rats (Miyata *et al.*, 1991b), indicating that 5-HT-induced increases in fecal pellet output in rats is mediated by 5-HT$_3$ receptors. YM060, a potent and selective 5-HT$_3$ receptor antagonist, and granisetron inhibited this response to 5-HT. Methysergide, however, a 5-HT$_1$ and 5-HT$_2$ receptor antagonist, did not affect 5-HT-induced defecation. 5-HT$_3$ receptors are widely distributed in the central nervous system (Kilpatrick *et al.*, 1988) and peripherally, particularly on vagal afferent terminals (Richardson *et al.*, 1985; Round and Wallis, 1986; Ireland and Tyers, 1987). The effects of 5-HT through 5-HT$_3$ receptors are considered to be mediated *via* the release of acetylcholine from nerve terminals. Because atropine and tetrodotoxin also inhibited 5-HT-induced increases in fecal pellet output, these results support the hypothesis that the effects of 5-HT are mediated through 5-HT$_3$ receptors.

TRH is one of the endogenous substances that centrally mediate several stress-induced responses of gastrointestinal function (Diop *et al.*, 1991). Central injection of TRH has been reported to influence gastrointestinal contractility (Tonoue and Nomoto, 1979; Garrick *et al.*, 1987), intestinal transit (Horita and Carino, 1982; Horita *et al.*, 1985; Carino and Horita, 1987) and gastric emptying (Gue *et al.*, 1987; Maeda-Hagiwara and Tache, 1987; Bueno and Gue, 1988) in rats, mice or rabbits, whereas the s.c. injection of TRH in the present study resulted in an increase in fecal pellet output in rats. Concerning the mechanism by which TRH may affect bowel function, it has been reported that TRH acts centrally to induce vagally dependent cholinergic (Garrick *et al.*, 1987; Basso *et al.*, 1988) and serotonergic (Horita and Carino, 1982; Horita *et al.*, 1985) stimulation of gastrointestinal secretory and motor function. In our studies, 5-HT$_3$ receptor antagonists (*i.e.*, YM060, granisetron and ondansetron), atropine and tetrodotoxin significantly inhibited increased fecal pellet output induced by TRH in doses that decreased 5-HT-induced fecal pellet output. These results comfirm that TRH acts in the central nervous system as a modulator of gastrointestinal function, at least of defecation, and that the effect of TRH is dependent on endogenous 5-HT released from the EC cells and/or enteric serotonergic neurons in the intestine.

Williams *et al.* (1987) have reported using partial wrap restraint stress model that endogenous CRF mediates stress-induced changes in colonic function. The action of CRF is centrally mediated through CRF receptors, and CRF-induced

alteration of colonic transit appear to be primarily mediated by vagal efferent pathways (Tache *et al.*, 1987; Lenz *et al.*, 1988). Diop *et al.* (1991) have suggested that the release of CRF in the central nervous system by cold stress promotes the release of TRH, which in turn directly controls the rate of gastric emptying. As mentioned before, TRH activates colonic transit *via* a vagally mediated serotonergic mechanism (Horita and Carino, 1982). Taken all together, it is suggested that CRF centrally released by stress mediates changes in colonic function through the peripheral release of 5-HT.

One of the most important findings of this study is that 5-HT_3 receptor antagonists inhibited not only restraint stress-induced increases in the number of stools excreted in fed rats but also stress-induced diarrhea in food-deprived rats. These results may be associated with an other report in which immobilization stress resulted in an increase in plasma and brain 5-HT concentration in rats (Sharma and Dey, 1981). Furthermore, atropine and tetrodotoxin also showed inhibitory effects on restraint stress-induced bowel dysfunction, suggesting the involvement of peripheral muscarinic receptors and interneuronal pathways. Therefore, the inhibitory effect of YM060 on stress-induced bowel dysfunction, *i.e.*, increase in fecal pellet output and diarrhea, seems to be attributable to its 5-HT_3 receptor antagonistic activity. Taking these data together, the following hypothesis may explain the effect of restraint stress on bowel function; namely, 5-HT exogenously administered [1) see figure 9) or released from the EC cells and/or enteric serotonergic neurons, 2) by stress exposure, 3) or TRH injection, 4) binds to the neuronally located 5-HT_3 receptors, 5) and modulates bowel function, 6) through the release of acetylcholine, and 7) from parasympathetic nerve terminals. Figure 9 shows a schematic illustration of the mechanism by which 5-HT, TRH or stress may affect bowel function as suggested from experimental data.

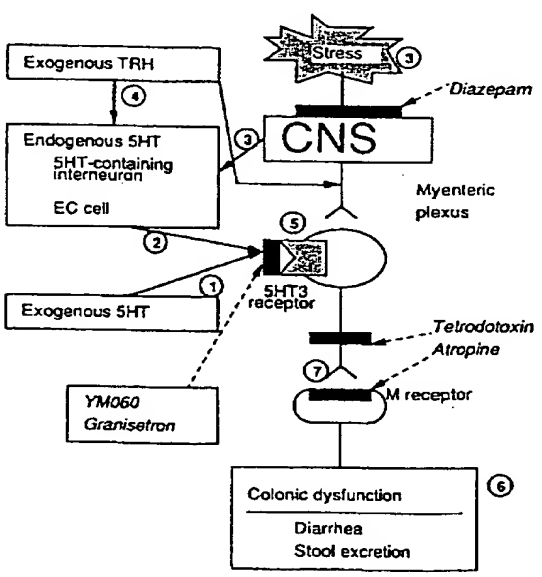

Fig. 9. Schematic illustration of the mechanism by which 5-HT, TRH or stress may affect colonic function as suggested from experimental data. 5-HT exogenously administered 1) or released from the EC cells and/or enteric serotonergic neurons, 2) by stress exposure, 3) or TRH injection, 4) binds to the neuronally located 5-HT_3 receptors, 5) and modulates bowel function, 6) through the release of acetylcholine and 7) from parasympathetic nerve terminals. CNS, central nervous system.

It was reported that 5-HT_3 receptors might be involved in the diarrhea responses to both antigen challenge and morphine withdrawal (Beubler *et al.*, 1984; Baird and Cuthbert, 1987). In humans, the role of 5-HT in regulating intestinal secretion is derived from the observation that ICS205-930, a 5-HT_3 receptor antagonist, ameliorates the diarrhea response in patients with carcinoid syndrome (Anderson *et al.*, 1987), a condition associated with the release of 5-HT (Ahlman, 1985). In the present study, YM060 and granisetron were able to inhibit 5-HT-induced diarrhea in mice, suggesting the involvement of 5-HT_3 receptors in their mediation. On the other hand, these compounds had no effect on PGE_2- and castor oil-induced diarrhea. Therefore, it was suggested that the release of 5-HT and/or 5-HT_3 receptors were not involved in the diarrhea responses to PGE_2 and castor oil. Because PGE_2 and castor oil have major effects on secretion and motility of the small intestine, the increase in fecal pellet output and diarrhea caused by restraint stress may be mainly attributable to the changes in colonic function.

In conclusion, restraint stress as well as exogenous 5-HT or TRH administration caused significant alterations in bowel function in rats. YM060, a potent and selective 5-HT_3 receptor antagonist, showed inhibitory effects on restraint stress- and 5-HT-induced increases in fecal pellet output and diarrhea and TRH-induced increases in fecal pellet output. These data suggest that endogenous 5-HT may mediate stress-induced changes in bowel function through the 5-HT_3 receptor.

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Send reprint requests to: Dr. Keiji Miyata, Medicinal Research Laboratories I, Central Research Laboratories, Yamanouchi Pharmaceutical Co. Ltd., 21 Miyukigaoka, Tsukuba City, Ibaraki 305, Japan.